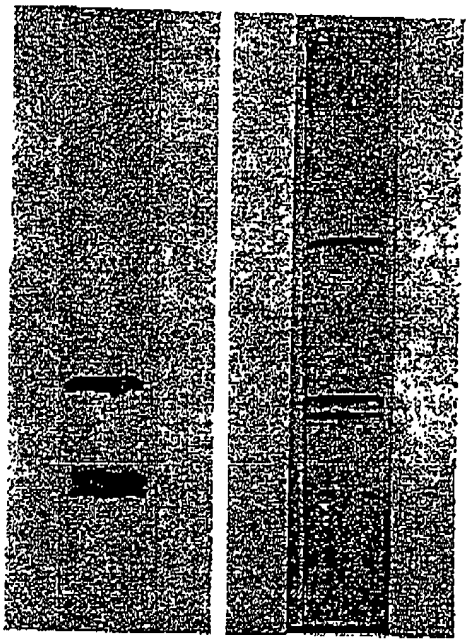
EXHIBIT 6

pH 8·0 (0·05 M Tris-HCl buffer) using 16 V/cm for 1 h. The two virus bands, located by staining a portion of the gel as described here, were cut out and the virus particles were eluted by vigorous stirring with 0·03 M sodium phosphate buffer, pH 7·6. Viruses from each band were examined with the electron microscope using negative staining with phosphotungstic acid, and found to consist of polyhedral particles, both of similar diameter (25–30 nm). In addition, the slow virus preparation contained small numbers of larger polyhedral particles (40–45 nm diameter).



a b

Fig. 1. Polyacrylamide gel electrophoresis in TAE buffer. Anode at top of gel. 6 mA/tube (internal diameter 5 mm). a, Unseparated *P. stoloniferum* virus (2·5 h) stained with amido black; b, RNA prepared from a (1·6 h) stained with acridine orange.

The two smaller viruses were serologically distinct. In gel diffusion tests, using the antiserum described here, two precipitin lines were obtained, both with the unfractionated virus preparations and with mixtures of the separated viruses. Separately the slow and fast viruses each yielded a single precipitin line. The two lines crossed over each other indicating non-identity of the two immunogens (Fig. 2). Examination of each precipitin line electron microscopically revealed the presence of only the smaller virus particles (25–30 nm diameter). The larger particles (40–45 nm diameter) did not give rise to a precipitin line with the antiserum used, and further investigation is required to determine if they are serologically related to either of the two smaller viruses.

Previous work has established that the viral RNA from *P. stoloniferum* is double stranded[2,3] with a sedimentation coefficient of 10–12S. Because this RNA was prepared without separation of the individual viruses it was suspected that it may have been a mixture. RNA prepared from the natural mixture of the three viruses by the phenol method[4] has now been examined by polyacrylamide gel electrophoresis (4 per cent acrylamide, 0·04 per cent methylene *bis*-acrylamide, TAE buffer) and found to give three principal bands (Fig. 2). Further characterization of the RNAs from the individual viruses is in progress.

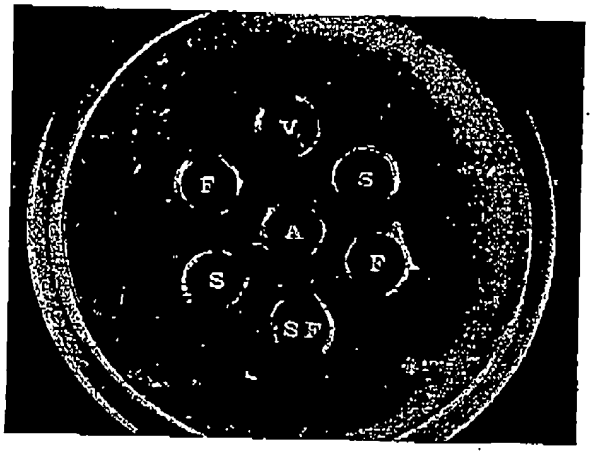


Fig. 2. Serological gel-diffusion plate. A, Antiserum; V, unseparated virus; F, fast virus; S, slow virus.

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Autoradiographic Detection of Molecular Hybrids between rRNA and DNA in Tissue Sections

We describe here a method for DNA–RNA hybridization which permits one to localize, at the microscopical level, molecular hybrids formed between a purified ^{3}H-labelled RNA species and DNA in the cell structures of tissue sections.

Chinese hamster cells of the CHEF 125 strain were grown in monolayer cultures in Eagle's minimal essential medium supplemented with 10 per cent calf serum. In order to label the rRNA to a high specific activity, ^{3}H-5-uridine (Radiochemical Centre, Amersham, 19 Ci/mmole) was added to the cultures at 30 μCi/ml. for three generations; a half-generation chase with non-radioactive uridine followed. For the same purpose the *Escherichia coli* 500 *Hfr U*− uracil requiring strain was grown for several generations in synthetic medium[1] in the presence of ^{3}H-5-uridine (10 Ci/mmole; 20 μCi/ml.); a hundred-fold excess of non-radioactive uridine was then added for about half a generation. Hamster rRNA was extracted from total cells and purified by sucrose gradient centrifugation[2]. *E.*

coli rRNA was extracted from cells, by grinding with alumina, following the same procedure.

The hamster ^{3}H-RNA (equimolar mixture of 28S and 18S components) studied here has been tested in conventional hybridization experiments with purified DNA extracted from hamster cells. The temperature and time of incubation (65° C for 14 h) and ribonuclease treatment of the hybrids (10 μg/ml. at room temperature for 1 h) were the same as those for the hybridization experiments with tissue sections. In these conditions, saturation was reached at an RNA concentration of about 2 μg/ml.; about 0·02 per cent of the DNA was hybridized at saturation. ^{3}H-rRNA from *E. coli* hybridized negligibly with hamster DNA, but the expected level of hybridization (0·2 per cent at saturation) was obtained with DNA from *E. coli*.

To minimize the effects of the fixative on the DNA structure, golden hamster brain and liver were usually fixed for 3 h in a mixture of absolute alcohol and chloroform (2 : 1); in some cases 10 per cent acetic acid was added. The tissues were then embedded in paraffin and sectioned at 5 μm. Following removal of the paraffin and hydration, the sections were mounted on slides with glycerin–albumin or collected on membrane filters (Millipore, *GS* type, pore size 0·22 μm).

Denaturation of DNA was carried out by heating the tissue sections, on filters or slides, in SSC/10 at 100° C for 10 min (SSC = 0·15 M NaCl–0·015 M sodium citrate). Annealing was then performed at 65° C for 14 h in 2 × SSC containing 3 μg/ml. of purified ^{3}H-rRNA from Chinese hamster (1,250,000 d.p.m./μg). From then on the sections on filters were treated as described by Gillespie and Spiegelman[3] for conventional DNA–RNA hybridization. The specimens were thoroughly washed by filtration with 2 × SSC and then treated with ribonuclease (10 μg/ml.) for 1 h at room temperature in 2 × SSC. After another washing, by filtration with 6 × SSC, the specimens were air-dried and prepared for autoradiography. For sections on slides the same procedure was followed except that the washing was performed by several changes of SSC solutions. After the hybridization procedure, the sections were air-dried and the filters glued on slides. All slides were dipped in undiluted Kodak *NTB* 2 emulsion, air-dried and exposed in a dry atmosphere at 0°–4° C for periods from 20 to 60 days. Processing was carried out with Kodak *D*-19 developer. The slides were usually stained with haematoxylin. The sections, attached directly to slides, were mounted with a coverslip. The membrane filters were air-dried and clarified with immersion oil.

Microscopic examination of the autoradiographs obtained in this way revealed that most nuclei were labelled. In brain sections this labelling was chiefly restricted to the nucleolar regions (Fig. 1*C*). Neural cells are particularly suitable for this kind of observation because of the considerable size of their nuclei and nucleoli (Fig. 1*A*). Similarly, liver cells showed a grain concentration in the nucleoli (Fig. 1*D*) but silver grains sometimes appeared also in other nuclear regions. This may result from the presence of several smaller nucleoli (Fig. 1*B*) in these nuclei which are known frequently to be polyploid. In all cases the cytoplasmic labelling was very weak or absent, especially in the case of sections on filters. This is probably because the washing by filtration is more efficient. On the other hand, the nuclear labelling was greater in sections mounted on filters, probably because of the binding of the denatured DNA to the filters which might result in a decrease in the loss of DNA from sections and in less renaturation of DNA. For these reasons and for its simplicity the filter method was preferred in our experiments.

To verify that the binding of ^{3}H-RNA to the nuclear structures was the consequence of specific DNA–RNA hybridization, a heterologous radioactive rRNA (*E. coli* ^{3}H-rRNA; 1,200,000 d.p.m./μg) was used in hybridization experiments on tissue sections following the method

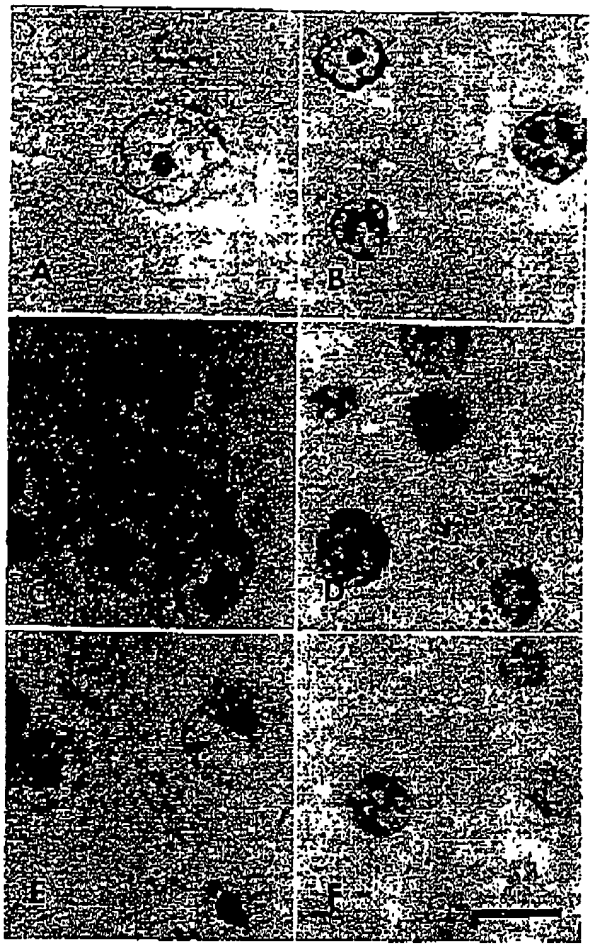

Fig. 1. Sections from hamster brain (*A*) and liver (*B*) fixed in alcohol–chloroform and stained with haematoxylin. Autoradiographs (exposure time 30 days) of brain (*C* and *E*) and liver (*D* and *F*) sections after hybridization with hamster ^{3}H-rRNA (*C* and *D*) and *E. coli* ^{3}H-rRNA (*E* and *F*). Fixation and staining as for (*A*) and (*B*). The bar represents 10 μm.

described. Fig. 1*E* and 1*F* demonstrate that there is no labelling in the cells. Further evidence for the specificity of the hybrids obtained in tissue sections comes from competition experiments. The annealing reaction was carried out as described but in the presence of non-radioactive RNA in a ten-fold excess with respect to hamster ^{3}H-rRNA. When the non-radioactive RNA was homologous (hamster rRNA) to the radioactive RNA, the labelling of the nuclei and nucleoli was drastically diminished, while the addition of non-radioactive heterologous RNA (*E. coli* rRNA) lowered the nuclear and nucleolar labelling only slightly.

In all the experiments we have described the ribonuclease treatment following the hybridization was performed at a concentration of 10 μg/ml. at room temperature for 1 h. The suppression of this step resulted in a net increase of the grain number in the nuclei and nucleoli; a ribonuclease concentration of 100 μg/ml. (1 h at room temperature) reduced partially (30–50 per cent) the labelling as compared with the 10 μg/ml. ribonuclease treatment. In experiments with *E. coli* ^{3}H-rRNA, even in the absence of ribonuclease treatment, the grain count was at background level.

If the DNA thermal denaturation step (100° C in SSC/10 for 10 min) is omitted some hybridization still occurs on alcohol–chloroform fixed tissues. It is possible

that the prolonged heating at 65° C during the annealing incubation is sufficient to loosen somewhat the DNA structure and to allow a certain degree of hybridization; a more likely explanation is that, during the histological procedure for paraffin embedding, conditions are such as to cause DNA denaturation. When there was acetic acid in the fixative solution, the hybridization seemed to be about the same with or without the thermic denaturation step, probably because of the denaturing action of acetic acid.

The number of silver grains found in hybridization experiments with hamster ^{3}H-rRNA averaged fifty per nucleus in 30 days' exposure time, when the 10 μg/ml. ribonuclease treatment was adopted. There is, however, considerable variability—from ten to ninety grains per nucleus—because only part of the nucleus is present in each section. These values are of the order of magnitude expected on the basis of the number of rRNA genes per cell, the specific activity of the ^{3}H-rRNA used and the efficiency of the autoradiographic method (about 100 grains per nucleus in 30 days' exposure time).

While this manuscript was in preparation two articles by Gall and Pardue[4] and by John, Birnstiel and Jones[5] appeared which describe similar *in situ* hybridization techniques.

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Rare Bases in Animal DNA

The rare bases which occur in animal DNA deserve special attention because their presence is directly relevant to questions concerning the cellular, subcellular and species specificity of DNA methylation. In animal DNA, the base 5-methylcytosine (5-MeCyt) is usually detected[1,2]. Numerous attempts to find N^6-methyladenine (6-MeAde)—which is characteristic of the DNA of various micro-organisms[3–6]—in animal DNA proved unsuccessful[2,6–9]. This may be accounted for by the fact that animal cells do not contain the enzymes required for methylating DNA at adenine residues[1–9]. Nevertheless, 6-MeAde and certain N^2-methylated derivatives of guanine have been recently discovered in appreciable amounts in bovine and human sperm DNA[10]. Our present knowledge on the specificity of DNA methylation in animal cells is not only scanty but also contradictory.

We report here a comparative study of the content of 5-MeCyt in the DNA of invertebrate and vertebrate animals belonging to different taxonomic groups. Further, we have studied the degree of methylation of the DNA in the sperm and different somatic tissues of the same animal. Special attention has been given to the detection of 6-MeAde in animal DNA.

DNA preparations from different cells and tissues[11,12] were hydrolysed (72 per cent $HClO_4$, 1 h, 100°) and bases were separated by paper chromatography as described earlier[4].

5-MeCyt was found in the DNA of all animals studied regardless of their taxonomic position (Table 1). The DNA of *Helix pomatia* is somewhat different from that of other molluscs[7] with respect to the content of 5-MeCyt. DNA preparations from the sperm cells of various species of sea urchin differ: the quantity of 5-MeCyt in the DNA of *Strongylocentrotus intermedius* is three-fold less than that in the DNA of *Echinus esculentus*[1]. The content of 5-MeCyt in the DNA of various species of mammals (somatic cells of rabbit and bull) also seems to be different but not so markedly. It is of interest that the DNAs of the rodents studied (rat[1], rabbit, mouse) are similar and have lower 5-MeCyt contents as compared with other mammals. A large proportion of 5-MeCyt is specific for fish DNA (about 1·5–2 moles per 100 moles).

Table 1. CONTENT OF 5-METHYLCYTOSINE IN DIFFERENT ANIMAL DNA

Source of DNA	Moles per 100 moles 5-MeCyt $\bar{N}$	σ	n	G+C+ 5-MeCyt
Invertebrates				
Porifera				
Suberites domuncula: whole animal	1·8		3	39·5
Coelenterata				
Metridium senile: whole animal	0·9		4	39·0
Mollusca				
Helix pomatia: liver	0·6		5	41·0
Echinodermata				
Strongylocentrotus intermedius: sperm	0·6		4	41·4
Vertebrates				
Osteichthyes				
Cottus sp.: sperm	1·7		4	41·0
Trichiurus japonicus: sperm	1·5		3	41·3
Spheroides sp.: sperm	1·5		3	41·0
Cyprinus carpio (carp): liver	1·41	0·02	7	38·6
sperm	1·17	0·02	7	38·6
Oncorhynchus gorbuscha (salmon): muscles	1·60	0·03	9	44·0
liver	1·13	0·06	9	44·1
kidney	1·98	0·03	9	44·0
spleen	2·05	0·06	9	44·0
sperm	1·64	0·04	6	44·0
Amphibia				
Rana temporaria: liver	1·6		3	41·0
red cells	1·5		3	
Reptilia				
Testudo horsfieldi: liver	1·5		4	44·6
Mammalia				
Mouse: spleen	1·0		4	42·2
liver	1·1		4	42·2
Rabbit: liver	0·91		4	42·6
kidney	0·93		4	
spleen	0·88		4	
Pig: kidney	1·20	0·07	7	44·0
spleen	1·10	0·11	8	44·1
sperm	0·77	0·03	8	44·1
Sheep: liver	1·13	0·09	6	42·6
kidney	1·15	0·05	6	42·6
spleen	1·07	0·06	8	42·4
sperm	0·76	0·02	6	42·0
Bull: liver (bull I)	1·40	0·10	6	44·0
kidney (bull I)	1·30	0·07	6	44·0
lung (bull I)	1·32	0·02	6	44·0
spleen (bull I)	1·66	0·02	6	44·0
spleen (bull II)	1·39	0·07	6	44·1
spleen (bull III)	1·07	0·02	5	44·0
kidney (bull II)	1·34	0·02	6	44·0
sperm	0·76	0·06	11	44·2

$\bar{N}$ = Mean or $(\bar{x})$; σ = standard deviation or $s(x)$; n = number of determinations.

We observe that the content of 5-MeCyt of DNA varies from 0·5 to 2 moles per 100 moles depending on the species. Taking into consideration that some insects and Crustacea have about 0·1 mole per 100 moles of 5-MeCyt in their DNA (refs. 1, 2), it may be stated that in general the degree of methylation of animal DNA differs more than twenty-fold. This favours a marked species specificity in the methylation of animal DNA. As a rule, the more distant the species are taxonomically, the greater is the difference of the proportion of 5-MeCyt in their DNA. Animal DNA is less methylated than that of higher plants[13], but in animal DNA the variability of 5-MeCyt content is much greater within a particular species. In animal DNA, 5-MeCyt occurs predominantly in Pu-m^{5}C-Pu or Pu-m^{5}C-G sequences[2,14] and is evidently concentrated chiefly in GC-rich DNA. The possibility that the observed differences in 5-MeCyt content may be ascribed not only to the specificity of DNA methylases but also to the